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Morning Session

Moderator: Steve Shire

Panelists: Mary Cromwell (Genentech, Inc.), Ed Moore (Baxter Health Care Corporation), Ted Randolph (University of Colorado, Boulder), and Amy Rosenberg (FDA)

Editor's note: The transcript of these discussions has been edited to eliminate irrelevant conversations and to improve readability.

Unidentified Participant#1(Amgen): This question is addressed to Ted Randolph. What about the role of excipients in the formulation? Can we design excipients to competitively adsorb on surfaces to prevent the proteins from adsorbing and how would this and other specific factors actually affect the time scale for adsorption?

Ted Randolph: Well, part of the answer to that is that if you write a letter of support for my newest NIH grant [laughter] we might be able to answer some of those questions. But there is an interesting effect, actually, which is that those things that stabilize protein conformation that you might think about adding as a formulation excipient, for example, sucrose or trehalose actually can, if you look at the math of how things go to surfaces, tend to destabilize proteins on surfaces. And so we may have one of these nasty tradeoffs here where we have to balance bulk stability vs surface stability in order to make these things work. We are just learning, though, about how to really formulate against surface damage beyond the obvious, which is adding some surfactants to keep things off; but again, it is well known that adding things like Tweens tends to destabilize the conformation of many, many proteins, lowers their free energy of unfolding, tends to increase your level of soluble aggregates for the benefit of decreasing the level of insoluble aggregates. So there are a lot of nasty tradeoffs here that may come into play limited by our understanding of what is really going on at surfaces still.

Unidentified Participant #1: And what about the time scale for adsorbing and totally preventing it and, you know, keeping it away from our scale of manufacturing or stability storage?

Ted Randolph: Well, the adsorption is usually very, very fast, I mean, fast being a few minutes. And then the next question is, is there exchange with stuff in the bulk and stuff on the surface and at what time scale does that happen? I think the answer probably in most cases is going to be that if you are worried about protein adsorption, if you have something that effectively competes for that surface, even though you may have some proteins adsorbing and desorbing, as long as they do not unfold when they hit the surface, then you are probably okay because you will never have enough of a surface concentration of protein to cause problems. But that is probably again one of these things where the safest answer is to say that it is going to depend on the personality of your protein.

Unidentified Participant #2: Hi, Ted. Thank you for your talk, and I had a question for you. I am trying to put together the understanding of sodium chloride on this particular protein system and if I understood you correctly, sodium chloride was helping to destabilize the conformation of that protein; but from the data that you presented, that is not entirely clear to me because you have situations where you can have interactions with the native protein and you can have interactions with partially denatured protein, a la Timasheff, with different solvent systems and I know you are very familiar with that. So are you clear on the understanding that sodium chloride is in a formal sense destabilizing that protein, or is there something else in the formulation, for example, F68, which is helping to destabilize the protein the way you showed the urea and the guanidinium hydrochloride are destabilizing the protein and then the sodium chloride has the opportunity to interact with that partially denatured state. Is that clear?

Ted Randolph: Yeah, the sodium chloride effect on urea-induced denaturation curves is independent of whether there is Pluronic there or not. And in that particular case for that particular protein which is sort of unusual, it does not actually aggregate when you unfold it. You can even do thermal studies on it. Most proteins do not behave so nicely as this one in the bulk solution. So this is one of the unusual ones where essentially you just get a shift to the left of the free energy curves without many other cascading effects. But

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under normal situations, yeah, you worry about all kinds of multi-interactions with those things.

Unidentified Participant #3: Yes, what I am trying to do is parse the experimental system that you used. I mean you do not have urea or guanidinium hydrochloride in there, you have the F-sixty-eight (F68), so does this relate to real life or not?

Ted Randolph: A good question. You know, I mean, if anytime you make a measurement of a protein's free energy of unfolding, you are moving away from the real life situation whether you are heating it up to seventy degrees, you are adding a chaotrope, or you are using high pressure, and so there is a question. You are always doing an extrapolation back down to zero urea or lower temperature or lower pressure and making an assumption that things do not change as you are doing that, and I am sure there are cases where that is a bad assumption.

Marc Sutter (Ultrecht University): I also have a question for Ted Randolph. I recall that in the beginning of your talk, you said that those rare aggregates that you see, they grow with time and in the end you said the system is self-limiting because you have the particle of silica and then you have a coverage of protein and then it basically stops. So, is there something more to it than just what you presented?

Ted Randolph: Let me rephrase that question, "when was I lying?" [laughter]. By self-limiting, what I meant is that essentially even though those particles do indeed grow slowly, but they never form more than say half a percent or less, probably a tenth of a percent of the protein, even over a two- or three-year stability study. So after three years of a stability study, you will measure a few tenths of a percent of aggregate. They are all in the form of a few very large particles. So, by self-limiting what I meant is it does not create a shower or cascade of new nuclei that form and so essentially you can spin out the solution, collect the particles and there is almost nothing there; but you can see them quite clearly because they scatter light because they have gotten long.

John Wang (Genentech): A question for Amy. Is it the agency's current belief that the Eprex incidence is truly caused by the leachable BHA or BHT? And, you know, in many of our products we do see BHA, BHT in our current products, so, how much do you think you will be concerned if BHA and BHT are found in the drug product solution?

Amy Rosenberg: I am sorry, what compound are you talking about as being….

John Wang: The question is, is it the agency's current belief the problem with Eprex….

[Further exchanges to clarify the question were removed]

Amy Rosenberg: It pertains to byproducts of the vulcanizing agent.

John Wang: Right.

Amy Rosenberg: Yeah, well, I can say that to our knowledge we certainly do not have any reason to think that it is other than what the company's investigation has revealed. Eprex was never marketed in the Unites States, so we do not have as good a handle on those issues as do European regulators for instance; but, the companies make many public presentations and those are the data, at least those are the data presented. So, inasmuch as that is what we have to evaluate, we have—everybody went through the stage of thinking that it was the micelles and that the Epo and Eprex had inserted itself into the micelles. Everybody worried about denatured protein, products sitting on hot tarmacs, or problems with the cold chain. And those are worries but they are worries for all protein products, and I do not know that we have any information that would indicate that those were a particular worry for Eprex. So, we are limited in terms of having the same information that you have.

Unidentified Participant #4: (from Regeneron) A question for Ted. As you suggest, if I understand you correctly, to put a silicone particle as a stress test. What is the particle range of concentration? And everything is dose-dependent, I suppose?

Ted Randolph: That is a good question in that I do not have a good idea except that one can, with any stress test, you often try to sort of turn up the stress until you see an effect and so you can do a thermal test and for some proteins thermal stress may mean incubation at thirty degrees and for others it may mean incubation at forty-five and for others it may mean sixty; and I think probably the same thing would come out eventually as we look at things like adding exogenous particles. One perhaps good indication of that or a balance point might be to add enough of your particle so you would have a significant fraction of the protein that could be adsorbed on a surface if it formed a monolayer, as sort of a rule of thumb. So that depends on the size of your protein, and the formulation conditions in terms of concentration that you are operating at. What we found in this particular study were that weight ratios of silica particles to protein of about the one-to-one range enabled us to see this effect. But again, a different protein and a different size in particular might require different levels.

Unidentified Participant #5: My first question is to Mary. You mentioned in one of your case studies where ultrafiltration and diafiltration caused particulate formation and cloudiness that after filtration, the solution becomes clear again. Did you see a substantial yield loss during the ultrafiltration and diafiltration process?

Mary Cromwell: No, there was actually no apparent yield loss. There was a very, very small amount of protein that was precipitating during the UF-DF process. So, when we measured the protein concentration before and after, it was an insignificant amount of protein. Your eyes are just very sensitive to seeing a small number of particulates.

Unidentified Participant #5: Or, it could be that these particulates are not actually protein-related.

Mary Cromwell: No, they were definitely protein-related. We did do that analysis, but it was just a very small amount of protein.

Unidentified Participant #5: The second question is to all the speakers about the nature of the aggregates. Do you believe that all the aggregates are irreversible or actually there are two populations; some of them are reversible whereas others irreversible? Thank you.

Mary Cromwell: I will go ahead and start. We are seeing everything. In some cases we have very discreet self-associations that are occurring that are negative proteins interacting with each other down to one of the cases I showed with a disulfide formation. Once you have a covalent bond, it is not a reversible aggregate. With the precipitates we are seeing, you can solubilize them but I really would not want to use the protein at that point after they had precipitated in the specific cases that I was showing.

Ted Randolph: Yeah, I would say the same thing that very frequently there is a reversible component and an irreversible component. The irreversible component might make sense, it might not even be the driving force for aggregation in the first place; but it is simply if you think about partially unfolded proteins sitting at extremely highly concentrations within an aggregate, it provides many opportunities for subsequent follow on reactions. We have seen things like dityrosine, for example, in aggregates and linking through covalent linkages that are non-disulfide-based; and at the same time, reversible exchange and things you can and sometimes you can change a pH condition or a temperature condition and resolubilize an aggregate rather easily and other times you cannot.

David Brandwein (3M Drug Delivery Systems): Question for anyone who would care to respond. If you consider a process like spray drying where you essentially go from no interface, no water-gas interface to almost infinite water-gas interface really quickly, I am wondering what are the mechanisms there, that can cause aggregation? Do the mechanisms really occur so quickly because this is a really fast thing? And lastly, what kinds of excipients are used under those conditions to stop aggregation?

Ed Moore: I think it depends on the protein that you are looking at, so it is hard to answer without knowing exactly which protein that you are dealing with. So, but just generally, we certainly have a very hydrophobic interface such as an air-water or a gas-water interface and so I would expect that you are going to see some aggregation, if the protein is particularly susceptible to hydrophobic changes, in other words so it is not greatly stabilized and so you are going to see changes in the protein maybe that would lead to aggregation, and some unfolding that could lead to aggregation. If the protein itself does not have a high helical content, and if it has a high hydrophobic content, you would probably end up seeing some unfolding and potential aggregation occurring. On the other hand, if the protein is particularly stable then that kind of an environment for a brief exposure may not have a particularly adverse effect on it. You know it is going to be an empirical approach because of the case-to-case nature of it. So as far as conditions go, I would think about changing the pH as being one potential—again, you want to look at the ionic content on the surface of the protein, so changing pH may have a positive effect on stability of the protein in that kind of an environment.

Tracy Chen (CuraGen): I have a question for Ted. Can you comment about the relationship between soluble aggregates and the visible particles that you see. Do you believe you need a little bit or minimal amounts of soluble aggregate in order to form these or there is no relationship at all?

Ted Randolph: In the particular case we looked at for this talk we did not see soluble aggregates but then again we used SEC, so I guess this afternoon will tell me why that was not a good idea.

Wayne Gombotz (Omeris): I guess this would be to Mary, Edwin, Amy, maybe Ted if you have dealt with proteins in the clinic. I wanted to get back to the specifications in the amount of aggregation allowed. And let us just talk about an IgG1 monoclonal antibody because as Amy said earlier, any protein is going to be different and it is going to be have different amounts of aggregate that can cause a different amount of immune response. But when you are going forward with a product and you are going into a phase I IND, you are not going to know much about the immunogenicity and you are going to have a protein that will have some aggregation there, ie, your antibody will probably have a certain amount of aggregation. I wanted to know what level you are comfortable with a new protein for a phase I IND? What level would the agency be comfortable with? What level would industry be comfortable with putting in an IND? And then as you move forward into phase III and commercial, what levels are you comfortable with? So, I guess I am asking what are the highest and the lowest levels you have seen in your experiences with antibodies that had been in products?

Amy Rosenberg: I might have to call on Michelle Jessen to answer that because….

Wayne Gombotz: Okay.

Amy Rosenberg: But I would say just to generally address the question, not specifically dealing with IgGs, that the most important aspect of assessing aggregation in product development is tying it to a clinical experience. And clearly whereas we would think that if you had ten percent aggregates with any protein, and of course, I should preface that by saying you would have to do a risk assessment. So, if it is an Epo product that is very high risk that if you neutralize it, patients are in deep trouble, we would want to see a product where even in early phase INDs the aggregate level is extremely low. However, I think there is some wiggle room with that in terms of other kinds of proteins which may be lower risk, certainly proteins that if you neutralized them, the patients are not going to suffer unduly. The efficacy may be hampered and that is a problem for some but generally it is not going to cause deficiency syndromes and such. There may be a little wiggle room but our sort of operating mantra for aggregates is, as little as possible, and that the manufacturing process should be aimed at reducing aggregates to as great a degree as possible because we do not know. And, animal studies can sometimes be helpful. Certainly, using transgenic animals the way they did with the human interferon-alpha transgenics where I think that that the tolerance or even neonatally-tolerized mice, the tolerance to the human factor might allow you to explore effects of aggregates. Those could be potentially very, very useful although the tolerance in animal transgenics is different than the natural sort of tolerance in humans. At least that gives you some sort of a view. But the important thing is to be able to tie a manufacturing history with a clinical experience. So to have a good sense of what level of aggregation and what kinds of aggregates are going to induce immune responses, you have to have a good assay or good assays in place for aggregates that really measure them well and you have to have a good immunogenicity assay in the clinic so that you can really detect it. But that is true with all product characteristics, the manufacturing history is tied to a clinical experience and that is really where we get our safety database.

Mary Cromwell: Actually I want to add something to that. We have had a couple of incidences where we have products that have perhaps a higher level of aggregate than we are comfortable with and in those cases, we do quite a bit of biophysical characterization as well as looking at our tox data pretty closely before we take those into the clinic.

Tom Scherer (MedImmune Vaccines): This question is directed at Ted. I was wondering if you could comment on the techniques you used to measure the unfolding at the interface of your silicone particles and how sensitive that technique is or those techniques are?

Ted Randolph: So the techniques that we used to look at whether or not the protein retained—in this case mostly secondary structures—were derivative UV spectroscopy, where we looked at second or fourth derivatives of the UV signal. And taking the derivative reduces the contribution from scatter so you can actually look at some of these systems that do scatter a bit of light and using UV. At that point.

Tom Scherer: That is in suspension now?

Ted Randolph: That is in suspension; yes, and then by IR as well. Now both of those techniques are probably not going to tell you that you had five percent unfolded and ninety-five percent folded for what you are looking at, so they are not extraordinarily high sensitivity techniques. They will tell you something about the bulk. But what we were able to do in the particular case of this protein is put it on monolayer coverage onto the surface of the beads or the silica particles and then look at that, and that retained its secondary structure.

Tom Scherer: So the silicone particles were looked at as an isolated system away from the bulk solution?

Ted Randolph: Yeah, you could essentially put those under non-desorbing conditions depending on pH and look at that away from the bulk.

Scott Fick (graduate student at the University at Buffalo): This is a question directed to Dr. Randolph. Normally, when they design protein formulations they choose excipients based on their ability to increase the Tm such that it can potentially interrupt aggregation. Considering the fact that you have observed in your studies that the nucleation phenomenon can cause aggregation, in your opinion is the screening of excipients based on their ability to increase the Tm a strong strategy? Will it be good enough to prevent aggregation like what you have observed?

Ted Randolph: Certainly, screening based on Tm is a useful strategy. It is a strategy that very often gives you the wrong answer especially when you deal with things like surfactants. Surfactants as a class very often lower Tms and often turn out to be essential in the formulation to prevent aggregation. So, the Tm screen screens mostly for conformational stability of your protein. It does not screen for colloidal stability and then it does not screen for surface interactions, and if that is where your problem lies, you may get the wrong answer. That said, I am not saying that it is not a useful technique, it is a very, very useful technique and should be used. It is just that it has to be used in conjunction with some other techniques.

Unidentified Participant #6: Just in formulating like, you said, there will be always a mixture of what do you call it, reversible and irreversible aggregates. So you will have a mixture of whatever the ratio is and then you add surfactants. You said surfactant sometimes reverse the aggregation. Do surfactants have any effect on irreversible types of aggregates or do they only work on the reversible type of aggregate?

Ted Randolph: I do not think we have ever seen a case where a surfactant helped on the irreversible kind of aggregation. The only times we have seen that a surfactant helped in reversing an aggregation was when there were surfaces involved and it was essentially helping to desorb things off of surfaces. Probably the other thing that we have seen is that again, that sometimes surfactants can increase the level of the reversible aggregates. One often sees an increased level of soluble aggregates in the presence of Tweens, for example. And so actually what is probably happening there is that the surfactant is helping to solubilize and keep those guys in solution thus giving you higher concentrations of these reversible dimer-, trimer-, tetramer-level species. Maybe Mary can say if that is any of her experience.

Mary Cromwell: I think what we have seen with my limited experience with the detergents is that when you have a non-ionic type of detergent which is generally what you are putting into formulations, I have seen cases where if we have had precipitation, we have essentially cleaned out all soluble aggregates with the precipitation event. And by putting some polysorbates in the formulation, we sometimes halt that precipitation so you do accumulate some of the more discrete species in that case. I would not call it necessarily reversibility. In the cases where I have known that protein self-associate and I have looked at the effect of polysorbate, there has been no effect on causing them to dissociate if I have formed an aggregate, but it does sometimes keep it from going to the next step.

Unidentified Participant #6: [question edited for clarity]. [Has] anybody tried to use other gases like nitrogen [vs air] when you mix and then see if the aggregation will be different?

Mary Cromwell: I do know of one particular case where we were having an issue with a protein that precipitated in process and one of the questions was, whether it was the air that had been used as opposed to nitrogen for that particular step. What we found actually was that there was no difference. That was a protein however that was not susceptible to oxidation. I think you have to look at your protein specifically and whether or not you have an oxidation problem to figure out whether or not the difference between using air vs nitrogen would actually make a difference.

Steve Shire: I just wanted to mention, I did not hear anybody discuss the fact that when you put surfactants into formulations it is often, used to control for aggregation in particulate formation that occurs as a result of air-water interface generation. That certainly is something that, we have seen time and time again. This can help a great deal.

John Beals (Eli Lilly and Co): Amy, I was wondering if you could maybe postulate a little bit as we are on the verge of doing a lot of engineering the molecules to bring them forth to the marketplace, make better molecules, what is the possibility of engineering out MHC II binding and thus mitigating immunogenicity issues with a protein and/or with its aggregates?

Amy Rosenberg: So, there are, as you know, several groups that study proteins and proteins from the perspective of trying to identify the peptides that are MHC Class II binding, that would bind to MHC Class II with a high affinity and therefore create a CD4 T helper cell epitope that we think certainly that most responses to, immune responses to therapeutic protein products because their IgGs are certainly the ones that are most troublesome require some sort of T cell help. And so there are groups that have identified those peptides and in some cases, like in the case of TPO those have been shown to be immunodominant. And, you know, that is a strategy that I think is certainly worthwhile exploring. However, you know, one MHC's immunogen might be another MHC's tolerogen and vice versa So, you also—those strategies are predicated on sort of common elements of most MHCs but it does not cover all MHCs so there is going to be some segments of the population that may not be covered by those strategies. I certainly think they are worth exploring and worth seeing whether or not you can engineer them out. We certainly should be able to develop animal models that would validate or justify that approach in humans. So, I think, overall, it is a worthwhile strategy to consider. I do not know yet that we have enough data that say this will work for sure.

Afternoon Session

Moderator: Steve Shire

Panelists: Steven Berkowitz (Biogen IDEC), Michelle Frazier-Jessen (FDA), Jun Liu (Genentech, Inc.), and John Philo (Protein Alliance Labs)

Mary Cromwell (Genentech, Inc.): This is a question for Michelle. Would you like to comment on the specification for aggregates if you have an approved product and there is a change in the route of administration or change in indication?

Michelle Frazier-Jessen: Yes that is actually a really good question. I think it is going to depend upon once again your product, the indication and the clinical data that you have. For example, if you have a product that is licensed for a cancer indication and you have a certain amount of data with regard to that and a certain aggregate profile and specifications and you have also your immunogenicity assay to consider and how reliable that assay is, I think that then you maybe go to a rheumatoid arthritis or psoriasis indication depending upon what your aggregate levels are and the specifications and how good your immunogenicity assay is. I do not think we have seen this yet but I know that we have certainly entertained this or discussed it within our group at least. I am not sure what that would entail but I do know that probably, my thinking would be that certainly this is a different patient population and you are going to need to readdress your immunogenicity profile, probably you might have to requalify your assay with that patient population in mind because you are going from maybe an immunosuppressed population to one that might be overly active. And then likewise for intravenous vs SC for example, once again it is going to depend upon the indication, and if you are going from a population that has a higher risk to a lower risk or vice versa, whether that would be acceptable. We know that I think this has been published or well maybe I will let Amy say it. Go ahead Amy because I am thinking. [Overlapping conversation] I know that for one product in particular that it is much more immunogenic if given SC but if you give it IV then not necessarily will the same events be observed. So, it is really going to be very product- and indication-specific.

Yong Wang (ImmunoGen): This question is for Dr. Liu. Using different buffer systems, what kind of impact did you have on the results that you obtained from analytical ultracentrifugation in terms of what is the percentage of aggregates you will get?

Jun Liu: I think one of the issues we are faced with is that with analytical ultracentrifugation, when you try to look at the very low ionic buffer conditions, they get into the nonideal environment. In that case, probably the quantitation will be impacted. But if you just run it in the normal condition, the high ionic conditions, in general the quantitations are actually quite reasonable. Does that answer your question?

Yong Wang: Yes, yes somehow. So no matter which kind of buffer you use, you have to use it in a high ionic strength so you will have the same result?

Jun Liu: Correct. Ionic strength actually is the key thing that causes the irregularities in the protein samples. And in general, you know, as long as you give enough ionic strengths in buffer conditions, the difference seen between the buffers actually is quite small from my experience.

Amy Rosenberg (FDA): So I have a question for folks from Industry. If, say a subordinate comes to you, and you are trying to develop a new formulation of a protein or you are putting it into a new container closure and you have no idea what effects might be on aggregate formation, what sort of strategy would you use in terms of the techniques potentially employable to look at that? So obviously, we have heard about the strengths and weaknesses of many of these techniques but it seems as if some are better for detecting certain types of aggregates that others. So, when you are going into an unknown, what would be the strategy you would use for deciding which techniques to employ to really fully characterize the aggregates that might be formed?

Steven Berkowitz: One thing I can say is, with the acquisition of our centrifuge, we now do that routinely for all the new things that come in from research or [through] acquisition or partnership, we are using the centrifuge routinely to look at aggregation. Taking it further, we do have some capability of obviously looking at light scattering also as a supplement to that. And I think as John mentioned in his talk, there is no one technique inasmuch as you can bring to bear it was helpful.

Amy Rosenberg: That is why I am asking you to tell me what you would use. Would you start out by, routinely, using size exclusion chromatography? Would you then go ahead and do a light scattering analysis to see whether or not if what you have done has engendered very high molecular weight aggregates that you might not see in size exclusion? Would you use a field-flow fractionation to look for fragments? What I am trying to get a sense of is what would be a really optimal use of all of these techniques that have differing strengths and weaknesses?

Steven Berkowitz: Maybe John can comment.

John Philo: Well, certainly, I agree. You would probably start off with your standard things and then go to others for example, for just a quick check to determine if are you suddenly generating some very large aggregates. Dynamic light scattering, for instance will take about an hour to do this in batch mode. It is very straightforward if you have got the instrument in your lab. So, that is just not hard to do.

Jun Liu: Yeah, I generally agree with John and Steve's comments. I think definitely the light scattering and AUC will be the method that you want to run. In terms of whether we are going to use Field Flow Fractionation (FFF) that really depends on whether that system you have is suitable for the FFF because from my experience, not all the aggregates can be separated well by the FFF. If you can separate them by the FFF, certainly FFF can be a very useful tool. And FFF also can connect with light scattering; it can give you additional information about some very large aggregate formations.

Steven Berkowitz: I have a question for John. In terms of doing dynamic light scattering, John, what are your thoughts in terms of when you get to big particles are we looking at dust or are we looking at the product? That is a problem.

John Philo: Well, yes. You see that is definitely true. That can be a problem. I mean generally speaking, what I tend to do is centrifuge the samples as a pretreatment because the particles that I am centrifuging out are really beyond the normal range of the instrument anyway so if they are in there, they are going to cause trouble. But yes, that is one of the drawbacks of the technique is when you see the particles; you have no idea what the chemical nature of those particles is. But also to go back to Amy's original question, part of what would guide me in what is right to do might depend on for example on what kinds of things have been generated under stress testing. If no matter how you beat up on the sample, you have never really seen any tendency to generate some of these really large things and, fifty to hundred nm then, you are probably not going to do it. I mean, like I say, it is not that hard to check so maybe you should do it but in general I think you should be guided by that as well.

Wayne Gombotz (Omeris): It seems like all these methods have some value to look at different types of aggregation but it seems like the method that everyone seems to fall back and running is SEC because it is pretty easy and you can validate it and you can train research assistants to run it and get it up and going in the lab. I am curious about these other methods and your thoughts on which ones you think would ever be able to be validated and run as a QC release test and do you envision any of these ever being run in a QC lab to release product? I guess I ask that to everybody up there.

Jun Liu: I think in the near future it will be very difficult for something like AUC and FFF to become a QC method. Unless you have some samples that you, really do not have any other way to analyze that, and then you probably want to go with AUC or FFF or even light scattering. And just like in a bioassay, you can have ±10 to 20% assay variations. You should be able to do these experiments in a QC environment; I think that is probably the way to go in the future. That is my opinion.

Steve Shire: John, do you have any comments?

John Philo: Let me briefly mention, certainly I am aware [that] there is at least one company that does size exclusion coupled with light scattering as a lot release. I believe that is a vaccine product. And I think perhaps somewhere dynamic light scattering might be used for lot release. In terms of training of technicians to do the measurements, I think that is fairly straightforward. The data interpretation might be an issue but, I think that can be worked out. Also just a turbidity assay as a go-no-go on aggregation I think is a very good one and very straightforward and could be a good QA QC release.

Steve Shire: Listening to a lot of discussion about the methodologies that are used and listening to the talks this morning, one thing that has occurred to me is we have a lot of tools at our disposal that can help quantitate or at least give profiles of aggregate distribution sizes but given what Amy had said, it seems like we have done less in the area of trying to figure out what these things actually look like. I know that it is a difficult job, especially spectroscopically. One of the things that I struggle while listening to your talk John, and maybe Steve as well, was, you know, in the old days, sedimentation velocity was used mainly for sedimentation coefficient analysis which could be done very precisely. I think that coupled with some of the hydrodynamic modeling programs it might be useful to try to start distinguishing whether the aggregates are forming a certain way vs other ways. And I would just get some input from you guys what you think about some of that type of methodology.

John Philo: All right, I will bite. Well, I think you are certainly right that we can do that. Our computer power is such now that in principle we can model some of these things and calculate sedimentation coefficients for different possible configurations. I think the problem is ultimately to convince yourself that this has any basis in reality. I would say that I try to pay attention somewhat to some of the smaller oligomers, and what are their sedimentation coefficients? Do they make sense hydrodynamically? There are a few cases where we think we have seen two different types of dimer in the same sample with clearly different shapes. So you can start to do some of this but the problem is how do you test what is ground truth on your models unless you have some imaging technique that you believe confirms that? I know some of the companies are doing some playing around with atomic force microscopy, perhaps I should not characterize it as playing around, but are being serious about it, but it is clearly developmental work, but again, typically there you are still putting it down on some sort of a surface thing to do it and tapping on it and, then you question whether you have altered what it looks like, I do not know. But the people who do that tell me, different aggregates look different. Some of them are real diffuse, some of them seem to have some characteristic structure. So, I think there is potential there. I think part of the problem is there is very little, information out there that is shareable on these.

Steve Shire: I suppose the other difficulty is even if you did accomplish that and you had preparations with let us say different populations, with one aggregate size but different shapes, what do you do with that? I mean unless you have some sort of clinical information to relate to that is always going to be the problem. But it just seems like it could be nice to start collecting some of that to see where that might go. I do not know, just a thought.

Jun Liu: Yeah, I think Steve asked a good question, if the degradation aggregate amount is very small and to characterize the conformation of these aggregates is going to be very difficult because we are not generating enough amount of the representative material to do characterization. I have seen some people actually use tandem, or what we call two-dimensional chromatography. What they did is using the size exclusion, they separated the dimer form first, and then they passed the dimer into another chromatography method. They were actually able to separate the dimer into three different kinds of dimer forms. This kind of approach could be useful in some cases, if you can separate them, you can connect it with the other spectrophotometer methods and do further characterizations.

Steven Berkowitz: I would like to just make one comment about this point you brought up Steve and that is, when looking at aggregates, dimer, tetramers that [you] maybe isolating are fine but when you get to the really big aggregates, isolating them I think you have to be careful about how what you do may change their size. I mean the whole stability of aggregates is a question itself. It is like you have a different product on your hand. What is it? Is it stability that I do measure? Does that reflect what I actually had initially? So, I think that is something you have got to keep in your mind. You might be dealing with something that is pretty unstable and then you go make measurements, it may not be what you initially had to begin with. So, it is a little bit of a tricky business.

Mark Staples (MicroCHIPS): This is a general question. Does anyone have a good idea on what to do about assuring that you do not have a stochastic process going on? For instance, especially with regard to nucleation-based effects, what if only one out of a hundred vials is subject to a type of aggregation that leads to some adverse immunogenic response? In a way it is similar to what we all face in terms of sterility assurance and I would just be interested in hearing any comments on that and how we deal with that analytically. Or from the audience?

Michelle Frazier-Jessen: I think that it has recently come up on our end; what is the appropriate amount to sample as far as some of these things? And, I think that is actually a really good question. I do not know that I have the answer for it. But it might be that, certainly, you need to be sampling probably in the beginning, in the middle, and in the end and the number of samples that you need to take maybe should not just be, one, maybe it should be more. I do not really have an exact answer for you but I think that is certainly something that we probably need to think about a lot more because we might be missing a lot of things.

John Philo: One quick comment on that is in at least one case I am aware of where I mentioned trying to track some of those issues down with the dynamic light scattering. I do not remember exactly what the incidence in the vials was, but it certainly was not one in a hundred and it certainly was far from every vial having "snow." Yet when we looked for the precursors, they were there at some level in every vial. The lot had been damaged in manufacturing and the bulk had damage. Only in certain vials did you generate enough nuclei to push you over the threshold so you saw the visible particles. But the precursors were in the bulk.

Unidentified Participant #6: I want to make a comment on your question. I am not sure I agree with your analogy of sterility and a spurious particulate formation because those are two different things. Having done sterile product development all my life, sterility assurance is a huge issue. I mean that is separate. That is life or death for a sterile product. On the other hand, spurious particulate formation, which having worked as a hospital pharmacist, the first and foremost thing that you want to make sure is if you have a lyophilized product or even a liquid injectable, you shake it and you make sure that it looks clear before you make an infusion solution or give it to a patient. So that is the basic principle now. Just like you can never assure a batch is sterile unless you destroy the batch and you test every single component on every single vial. In the same way you cannot really catch the spurious particulate formulation formation. That is the bane of our existence. And, I think you just have to rely that the end user is smart enough and they would look at the vial and make sure there are no particulates because you have already validated your process and you have submitted all your data and everything looks fantastic and then things do happen.

Michelle Frazier-Jessen: I just want to add, I think that if for example you have precursors that you know because you have done the proper developmental studies or you really understand your product, you have a good knowledge of your product and how it degrades, and you know that those precursors might potentially lead to aggregates or particulates or whatever, then that is something that can be of value maybe in determining your sampling size or even as in-process control or something like that. So I mean knowing as much as you can about your product and how it aggregates really can be very, very helpful. It might save you some aggravation.

Unidentified Participant #7 (Elan Corporation): I have been worried about issues of protein aggregation for a while and just like many of the people who are here it seems that we have been improving our understanding incrementally, but there has been nothing that has really come to the fore, maybe with the exception of analytical ultracentrifugation, within the last ten to fifteen years that has dramatically changed the situation. Maybe I am trying to oversimplify things but I am wondering if there are other techniques that might begin to play a role in putting us in a more secure state when we talk about protein aggregation. We seem to be very timid still when we deal with these issues. Maybe it is oversimplification but, as anybody looking at relaxation times with NMR or other techniques, John, you have just alluded to, you know, people who are starting to use atomic force microscopy. Are there other things like that? This is a question for the panel and for the audience.

John Philo: Not that I know of. [Laughter].

Unidentified Participant #7: No? So we are dealing with a mature field that is just so complex, that we just have to be patient and deal with each one on a case-by-case basis and not try to approach it from another perspective?

Michelle Frazier -Jessen: I think there are probably technologies that are out there that are being developed. We in this group [might not] be necessarily aware of them. And I think that the technologies that are out there, we are seeing some improvements in, for example in the ability to maybe use them as a lot release and maybe they are far away from that right now but I think that there a lot of groups that are working on bringing that to fruition. It is just going to take time.

Steve Shire: I'd just like to back what Michelle said; I think there are companies certainly that are working on other methodologies. In fact, when we were trying to decide what to present here, I think it is fair to say we went and tried to get a complete overview of everything that is possible. Well, we do not have enough time, we felt we should try to concentrate on some of the more established methods, if you like. I would suggest that if we all want to hear about some more far out ways that people are looking at this, we will think about a second conference. Someone who is volunteering to organize it? [Laughter].

Mary Cromwell: In talking about the cross-validation, we have sort of been dancing around how close do they have to be when you say they are cross-validated. Another way to look at it is how different can the different methods give results and you still say that your SEC works and that it is validated. That is for the whole panel. [Laughter].

Jun Liu: I think first of all, you have to establish what kind of assay variation exists. For the AUC, you want to know what kind of assay variation you have. and what is the range you are talking about? When you have that information, then you can compare whatever the result you have from SEC and AUC, and you see how comparable they are, how different they are, and that is going to be your start point. If there is a very significant difference there, then you have to do some investigation and try to find out what caused the difference. AUC is very useful to detect major changes. We are not talking about like 0.1 or 0.2 percent difference and if you see that difference, it is going to be very difficult to argue which method tells you the truth.

Michelle Frazier-Jessen: I think if they are the same, then life is easy but if they are not then maybe you need to look at a couple of methods and compare. As Jun said, maybe you need to do some investigations as to why. Maybe that will lead to you improving your SEC method. I think we have certainly seen that before.

Steven Berkowitz: I sort of want to make a little comment on this. This certainly goes back to my earlier days when I remember light scattering studies were done many years ago and people would want to calibrate or bring a reference standard into being and several round robin inter-laboratory studies were done with reference polymers and it is amazing they came back pretty different. It is a difficult task to set firm numbers, but I think we would all like to have that as much as possible. When I am looking at data from ultracentrifugation, AUC, five to ten percent agreement, it looks pretty good, if it is really closer it is great. But, you know, I do not start getting worried until I am ten or twenty or so percent or something like that, them I am really concerned. But those are just rough guidelines, quite frankly, but it is hard. It is maybe a little bit of a case by case but I think we would all like to have definitive numerology around that quite frankly. That is sort of my perspective on it.

Michelle Frazier-Jessen: It is nice to hear that Industry says case-by-case. [Laughter].

Ed Moore (Baxter Health Care Corporation): I had another question pertaining to this morning's session. Mary Cromwell showed us one of the proteins that she had, I think, described it as looking like and behaving like honey. So as you are trying to do analysis using the methods that you all have talked about, I am guessing a lot of the proteins that you have described in your presentations are pretty ideally behaved proteins. So have you ever had any experience with a protein that has a high viscosity or has some unusual behavioral properties and how do you analyze the aggregates that may form from that so that you can get a true representation? We were just talking about how you define truth but just some of these non-ideal colligative properties of proteins present unusual problems for these methods and how do you resolve those issues?

Jun Liu: Actually, we have put together a paper and also a poster. In one of the sections we discuss highly viscous monoclonal antibodies. One of the methods we found to be useful and actually for quite some time (by Allen Minton at NIH) is to use a preparative centrifuge. We have looked at a protein at a hundred milligrams per ml and asked the question if there are any interactions. I think one of the things you have to keep in mind is if you have a weak reversible interaction, to quantitate what percent of the protein is aggregate would be very difficult and it also is meaningless because it really depends on what concentration you are talking about, and what condition you are talking about. What is very important is the consequence of the interaction. Because some of the interaction will cause degradation, some interaction will cause precipitation, some interaction maybe have no impact on protein stability. So I think that is what we need to focus on, not just the percent of how much aggregate in that particular concentration.

Unidentified Participant #8: Can I ask a general question that if there are soluble aggregates or reversible aggregates which go away upon dilution, then what is the significance of having those? It is like a drug in a small molecule, which is stereospecific and both R and S, are equally active. Now some companies may make millions separating them and go on, and do a lot of different things but a racemic mixture is just as good as a stereospecific pure compound. So having aggregates which are reversible and, where the compound is potent so that when you dilute it and give it to the patient, it works fine, then having those reversible aggregates, is that problematic?

Steven Berkowitz: I will give you my personal thoughts. I think certainly the bottom-line is when you deliver it to a patient is there any problem quite frankly, but, that is at the very end. When I think of a product having reversible aggregation, I get concerned because I think, well you have heard from all the other talks, environmental changes are very critical and when you formulate, you are formulating in rather different environments and I am amazed by some of these formulations where there is very little supporting polyelectrolyte to suppress charges. So when you put that into a person and you think of that environment, the ionic strength, and the high concentration of protein, what is the implication of those aggregates especially if the dosing solution contains some of those reversible aggregates? And it is interesting in one case where we found reversible aggregation, I went to the bioassay people and asked them how you are conducting your assays. And, they are actually doing these in dilute buffer solutions. And I asked them could they develop this in plasma because by the dosing into that kind of solution, then perhaps we could pick up difference in activity that would be reflective of the aggregation. Now, it does not address the whole big issue of immunogenicity but in terms of potency, well, maybe but then again I think you have got also, as Steve Shire brought out, is the accuracy of those methods relative to five percent, ten percent can you see those in that kind of assay? But on a gross level, you know, do we see a difference? Instilling some kind of due diligence around that potential problem; but I think one of the bigger problems is these catalytic events that trigger some adverse conditions that are hard to assess in the laboratory.

Steve Shire: Just one train of thought: you were talking about IV administration, but what happens if you want to do this subcutaneously and you have got something that is let us say a hundred and fifty mEqs per ml and is very viscous and we do not know what is going to happen with the subcutaneous, that is another issue. Have you tried to pump something through a syringe when it is highly viscous? So, there are pharmaceutical issues too.

Michelle Frazier-Jessen: I just wonder you have your reversible aggregates but let us say you have some kind of an exposure or even over time sometimes can those reversible aggregates not become irreversible?

Jun Liu: Well, we actually have some examples and what we have found is that some of the reversible aggregates, actually, are a precursor of some kinds of the precipitation phenomena. The precipitation will form after we keep it at 2–8 degrees for three or six months. If you bring the precipitated sample to room temperature, it will eventually be clear. So, reversible interaction does have some impact from a pharmaceutical point of view. You need to invest to make sure that is not an issue for long -term storage.

Sue Richards (Genzyme): My question actually was related to this discussion that we are having here and that is, all this characterization around aggregation is all more focused around product quality and consistency and stability. And my question is, when you put that into a patient via SC or IV, does that mean that all bets are off or is it that what we see in the vial is a worst case scenario potentially? You know we know what plasma is like the viscosity, the pH concentration, and those kinds of things and are there any predictive models in place that could be used to help in that regard? So, I just wanted some comments from the panel in that particular area.

Steven Berkowitz: Well, I certainly think it helps to understand the physical chemistry of the molecule I mean as much as you can and what you have in the vial can again be very different from what the environment is going to be when you put it into a person and what you know about that molecule in terms of its physical chemical properties. How it responds could tell you a lot potentially, but obviously you would like to also in some way experimentally mimic those conditions as best you can in the laboratory but I think the point has been adequately made, that the environmental conditions have a tremendous impact especially for reversible aggregation and you need to consider those transitions from one environment, from a vial to the patient environment and how that plays out.

Mary Cromwell: So, I actually want to address the original question, which was about the reversible aggregates, and basically do you care if they are going to dissociate anyway when you inject them IV? I think one of the things you have to keep in mind is the kinetics of the dissociation because there is one case that we have had where the kinetics are fairly rapid though half-life is long so you can presume that the dissociation is going to happen over a fairly short time span relative to the circulating half-life. We have another instance where the dissociation is extremely slow where the circulating half-life is on the order of hours, the dissociation takes days to weeks. So there in that case even though it is reversible essentially it is worth the patient's concern. So I think that is a critical point that you have to take into consideration and again getting back to Susan's question about what happens in the body. In one case we have seen with one of our antibodies where we were considering SC formulation. We know that if we raise the pH and we raise the ionic strength, which would pretty much mimic the SC space and raise the temperature, we are going to get more aggregate. So there is a potential to form more even though it is reversible.

Amy Rosenberg: I guess this feels like a case of déjà vu all over again in that the same questions keep coming up every time we discuss aggregates and I am wondering if you are saying that you have some data on what the SubQ space does to injected proteins, soluble proteins. Why is this stuff not published? Why is it not being investigated? I mean who is waiting for whom to do the studies? It just seems to me it is time to do the studies, answer the questions so we can stop asking the same questions over and over again. So I mean, I think, maybe it would be reasonable to put a consortium together to start answering questions and maybe we will find out that everything is case by case and that you have to look at it and that is okay. But I think we have to maybe start trying to get some answers instead of asking the same questions all the time.

Unidentified Participant #9: Amy, I think what you just said is a more articulate way of asking the question that I asked before and I just wanted to add one thing. I put it in terms of technology but maybe what it is a series of parameters that would become universal as a way to describe both the process and the final end point for an aggregate state and if we could talk in terms of a unified set of parameters, it would clear the air and make people feel more secure about these processes.

Unidentified Participant #10: I just want to make a comment, John. I think there is something John, regarding your definition of soluble and insoluble aggregates, I think I can argue with that. I just want to make an alternative, what do you think of a term like visible and invisible precipitates, aggregates? [Laughter].

John Philo: Well that is fine. Most of my point is there certainly are a lot of different definitions of what is insoluble, okay? And if what your definition is that it does not pass through a 0.2-micron filter, then why not say that, okay? And if your definition is it is insoluble because when you put it in the microcentrifuge, it gets pelleted and you can describe that by a sedimentation coefficient and we can all agree what that means. So on that side, and on the other side it is just that there is such a range with insoluble aggregates that sometimes makes a difference and, if you are talking of dimer, trimer, tetramer, then let us just call them small oligomers vs bigger things. I just think when we have this specific information that it is useful to talk about it. And in part I am aware of this because, as I said, we deal with so many clients and every one of them means something different by these terms and we have to sort of go through this process of making sure we are not talking about this before we make any progress.

Steven Berkowitz: I have sort of one point to make on especially in analyzing these very big particles and some of these analytical techniques such as John said, there is dynamic light scattering, the actual size of the sample is actually pretty darn small and you know the uniformity of those particles might be an issue in terms of detection. I just want to get any comment about that.

John Philo: Yeah, well you can definitely see sampling errors for some of the big ones. The instrument I use requires twelve microliter samples so, you know, sometimes there is one big particle in there and the next sample it is not there.

Steven Berkowitz: Right, but the actual beam size of the scattering element is pretty small.

John Philo: It is even smaller, yeah. It is down in the nanoliter ranges. It is the volume you are really looking at. Well, definitely you can sit there and watch individual large particles wander in and out of the beam if they are in there.